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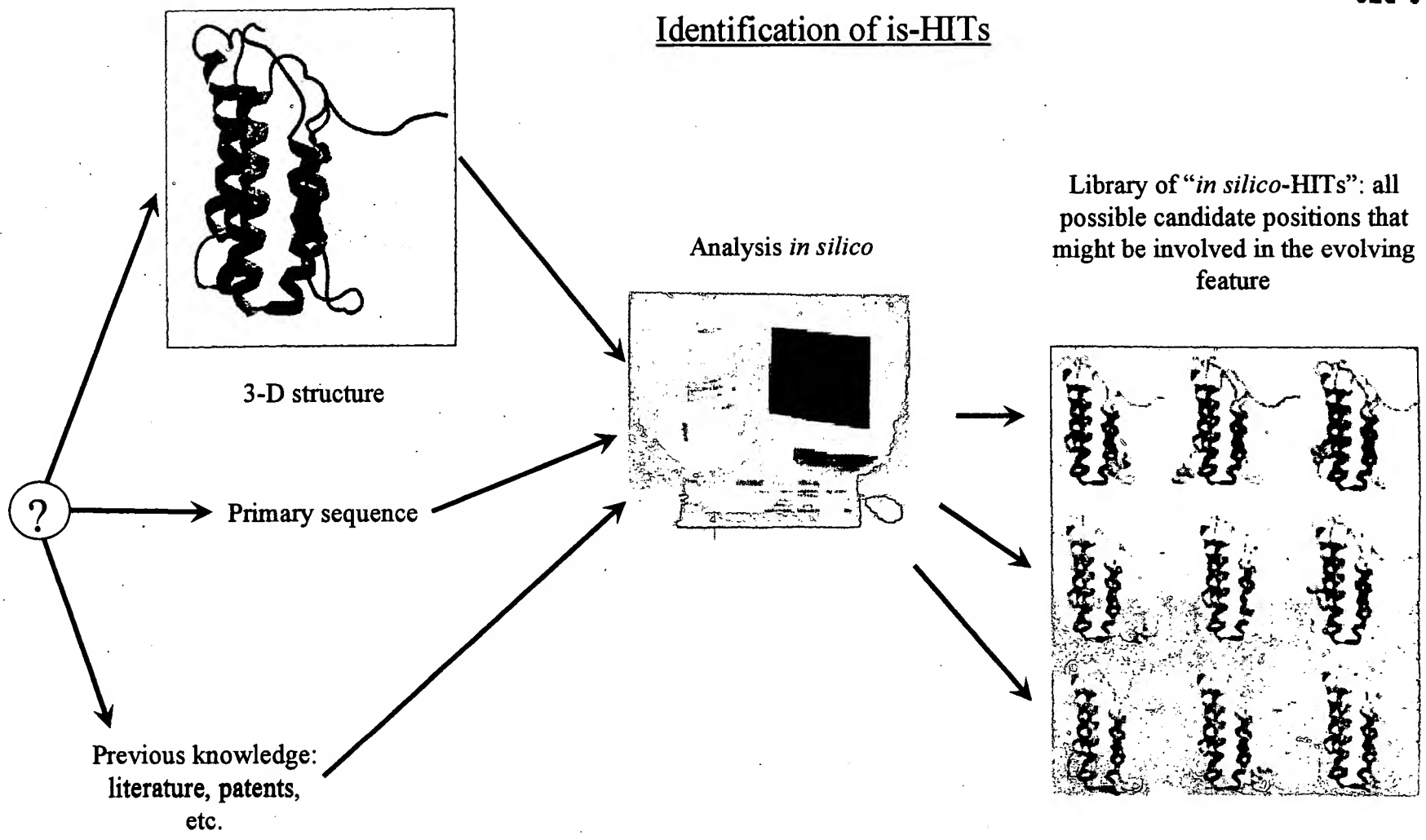

FIG.1A

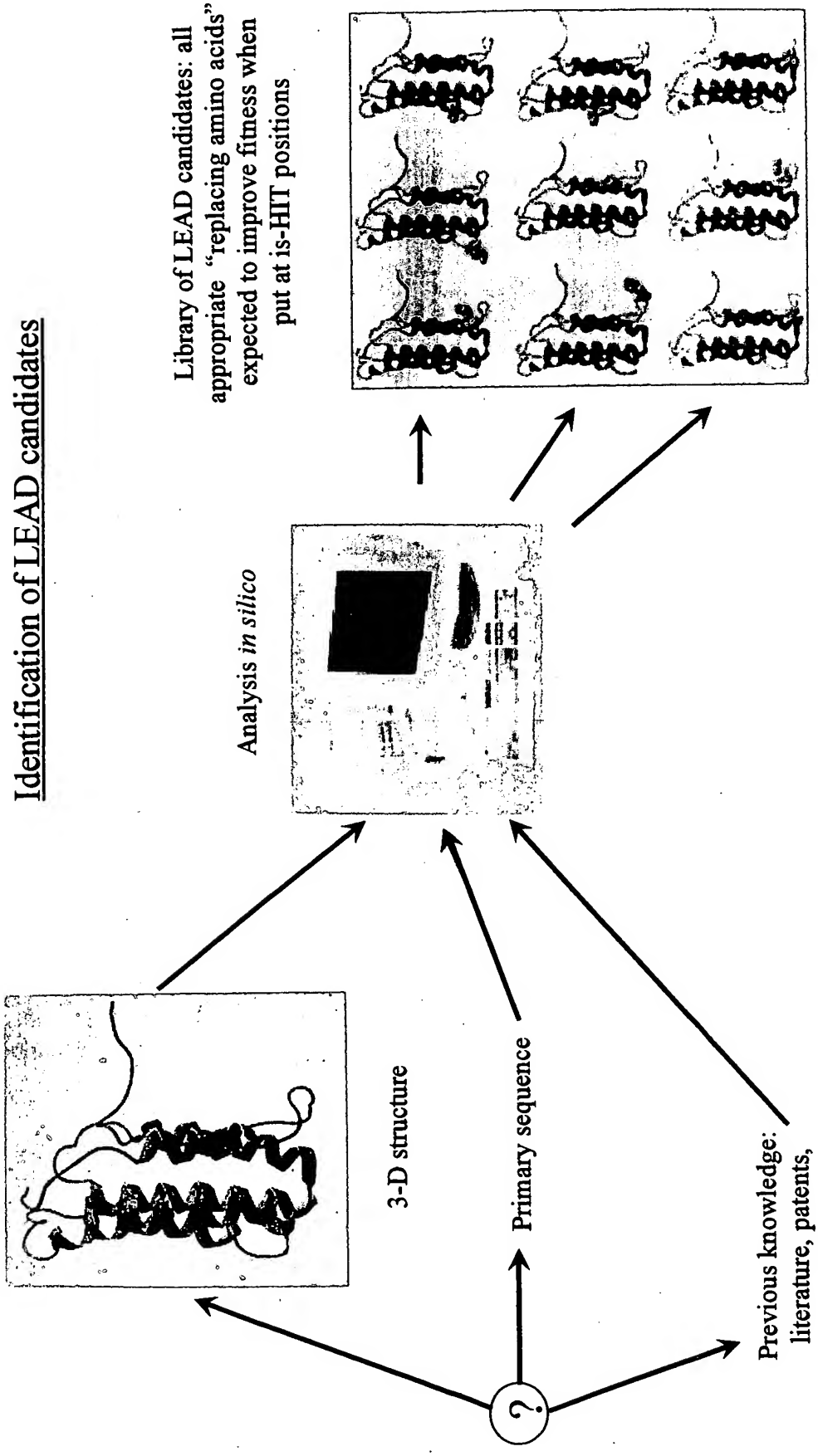
Identification of LEAD candidates
Library of LEAD candidates: all appropriate "replacing amino acids" expected to improve fitness when put at is-HIT positions
Analysis *in silico*
3-D structure
Primary sequence
Previous knowledge: literature, patents, etc.
?

FIG.1B

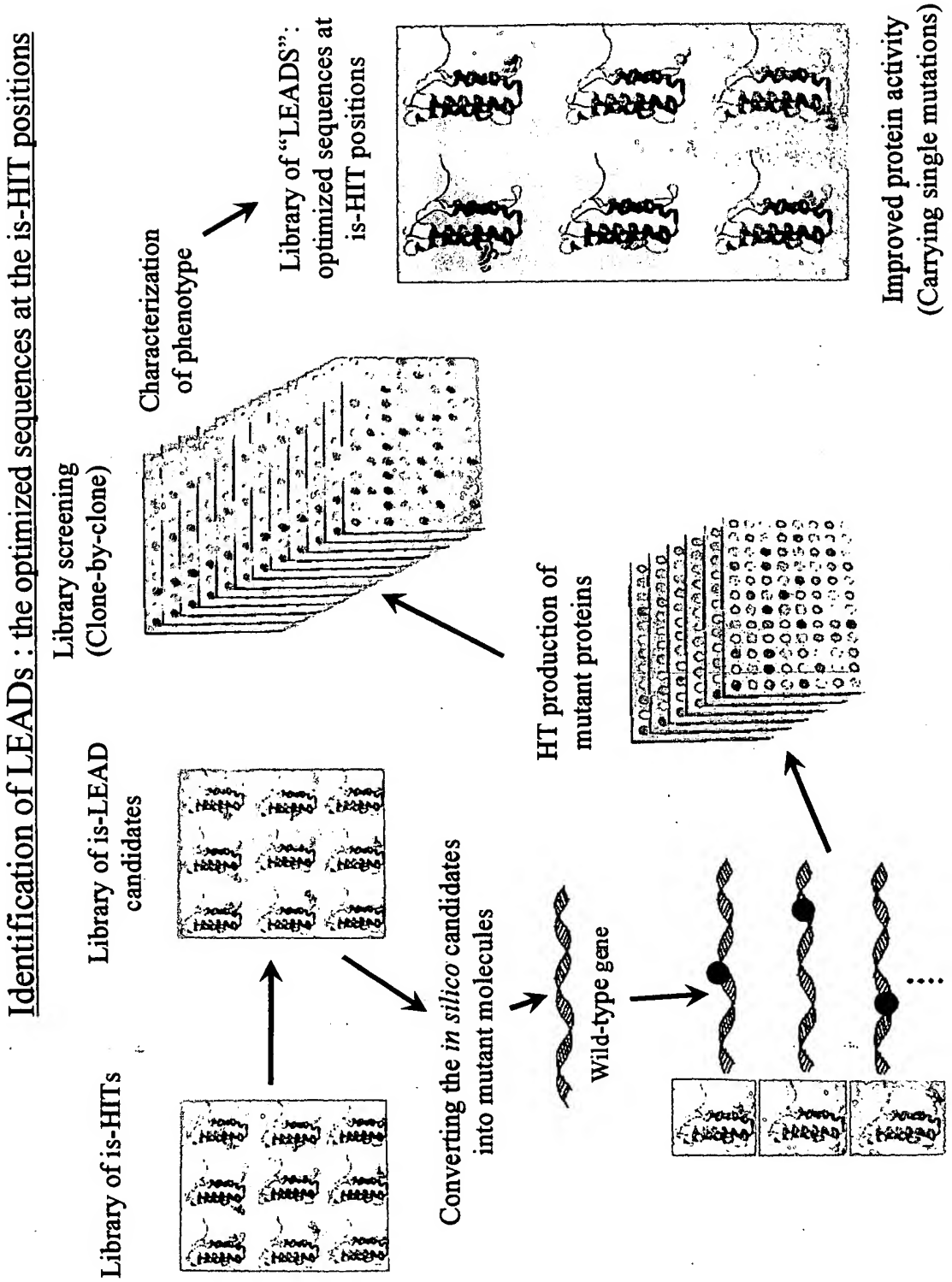
Identification of LEADs : the optimized sequences at the is-HIT positions
Library of is-HITs
Library of is-LEAD candidates
Library screening (Clone-by-clone)
Characterization of phenotype
Library of "LEADS": optimized sequences at is-HIT positions
Improved protein activity (Carrying single mutations)
Converting the *in silico* candidates into mutant molecules
Wild-type gene
HT production of mutant proteins
Library of mutant genes

FIG.2

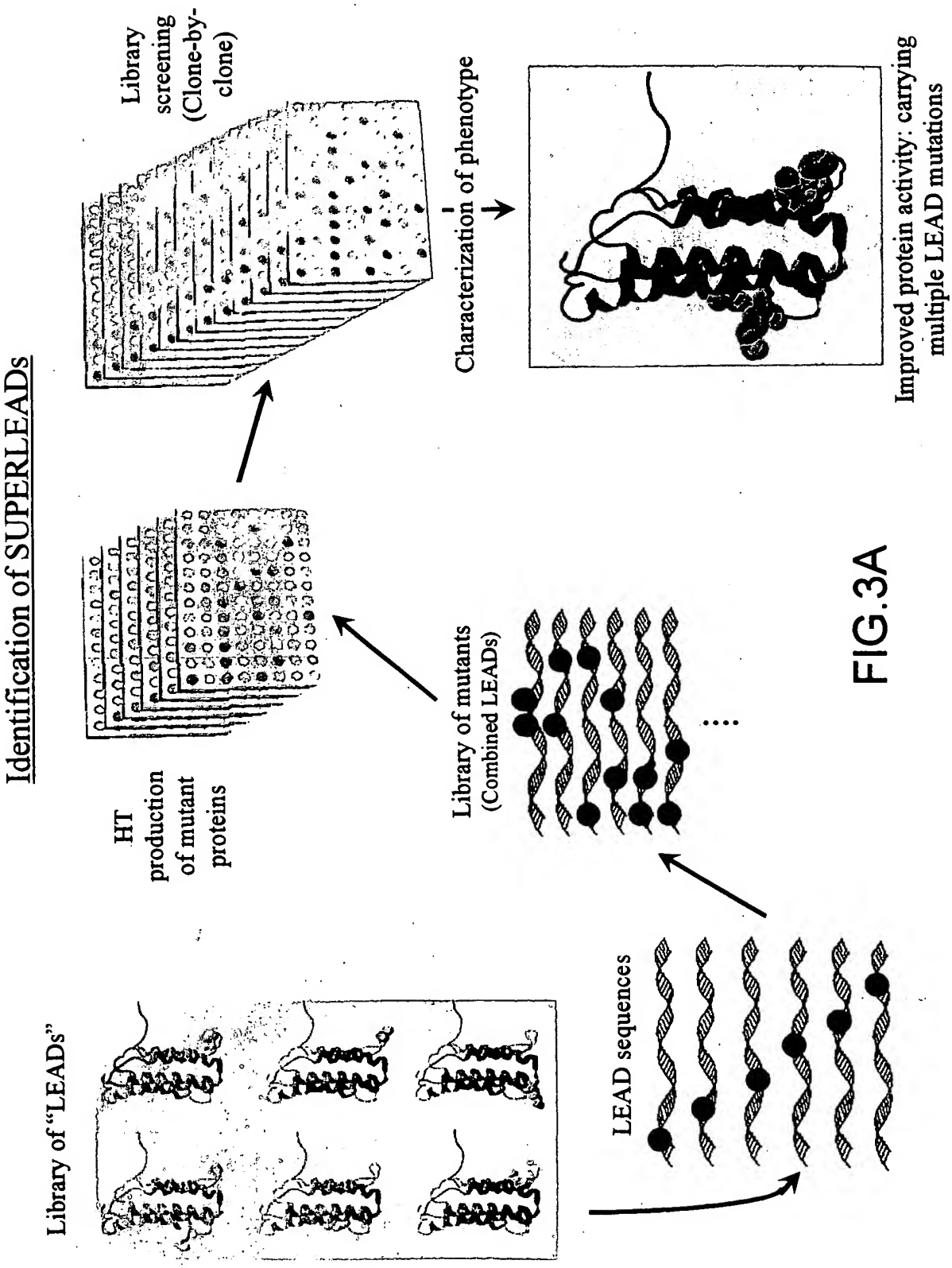
Identification of SUPERLEADs
Library of "LEADs"
LEAD sequences
Library of mutants (Combined LEADs)
HT production of mutant proteins
Library screening (Clone-by-clone)
Characterization of phenotype
Improved protein activity: carrying multiple LEAD mutations

FIG.3A

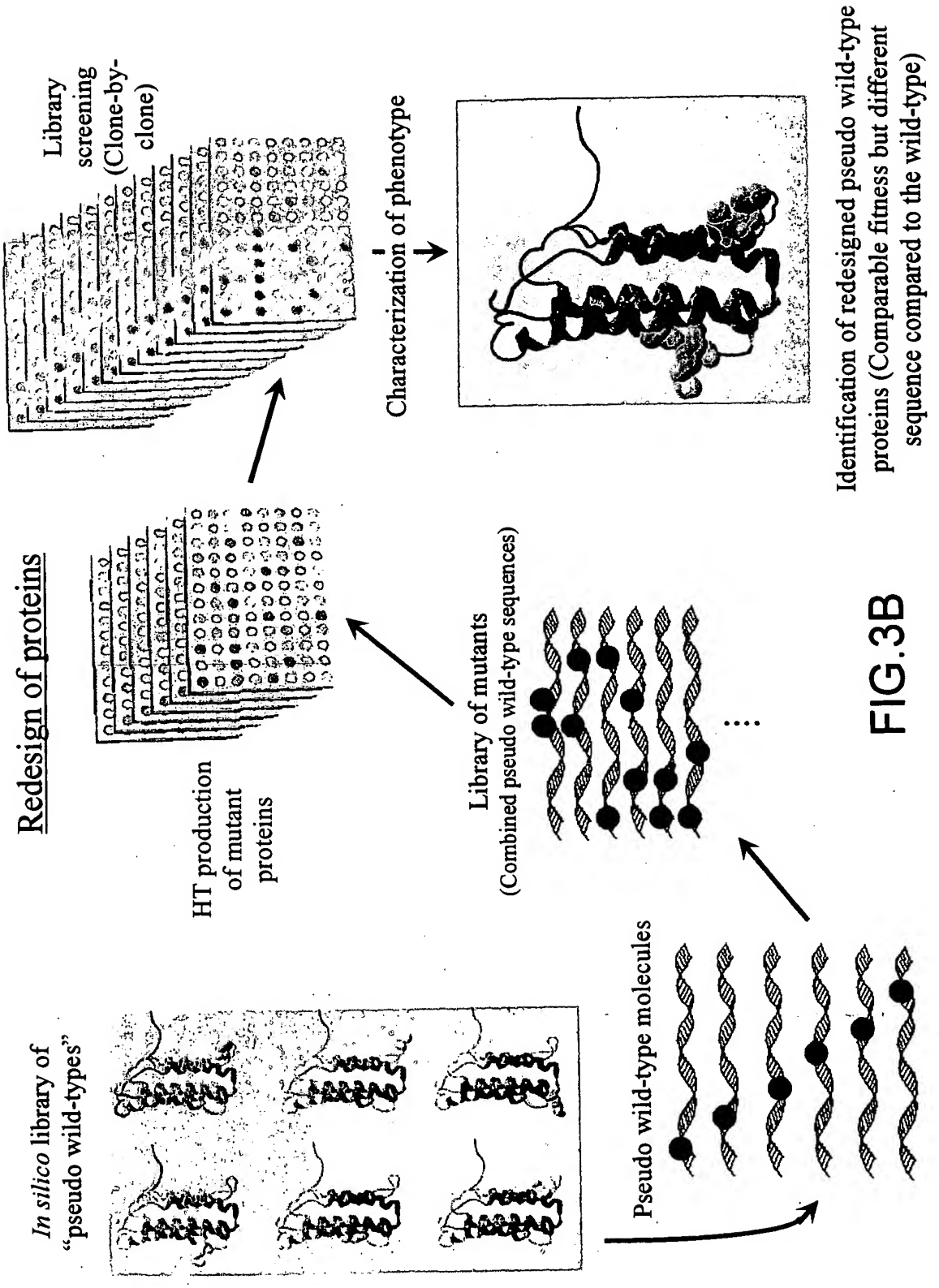
Redesign of proteins
In silico library of "pseudo wild-types"
Pseudo wild-type molecules
HT production of mutant proteins
Library of mutants (Combined pseudo wild-type sequences)
Library screening (Clone-by-clone)
Characterization of phenotype
Identification of redesigned pseudo wild-type proteins (Comparable fitness but different sequence compared to the wild-type)

FIG.3B

"Additive Directional Mutagenesis" (ADM)

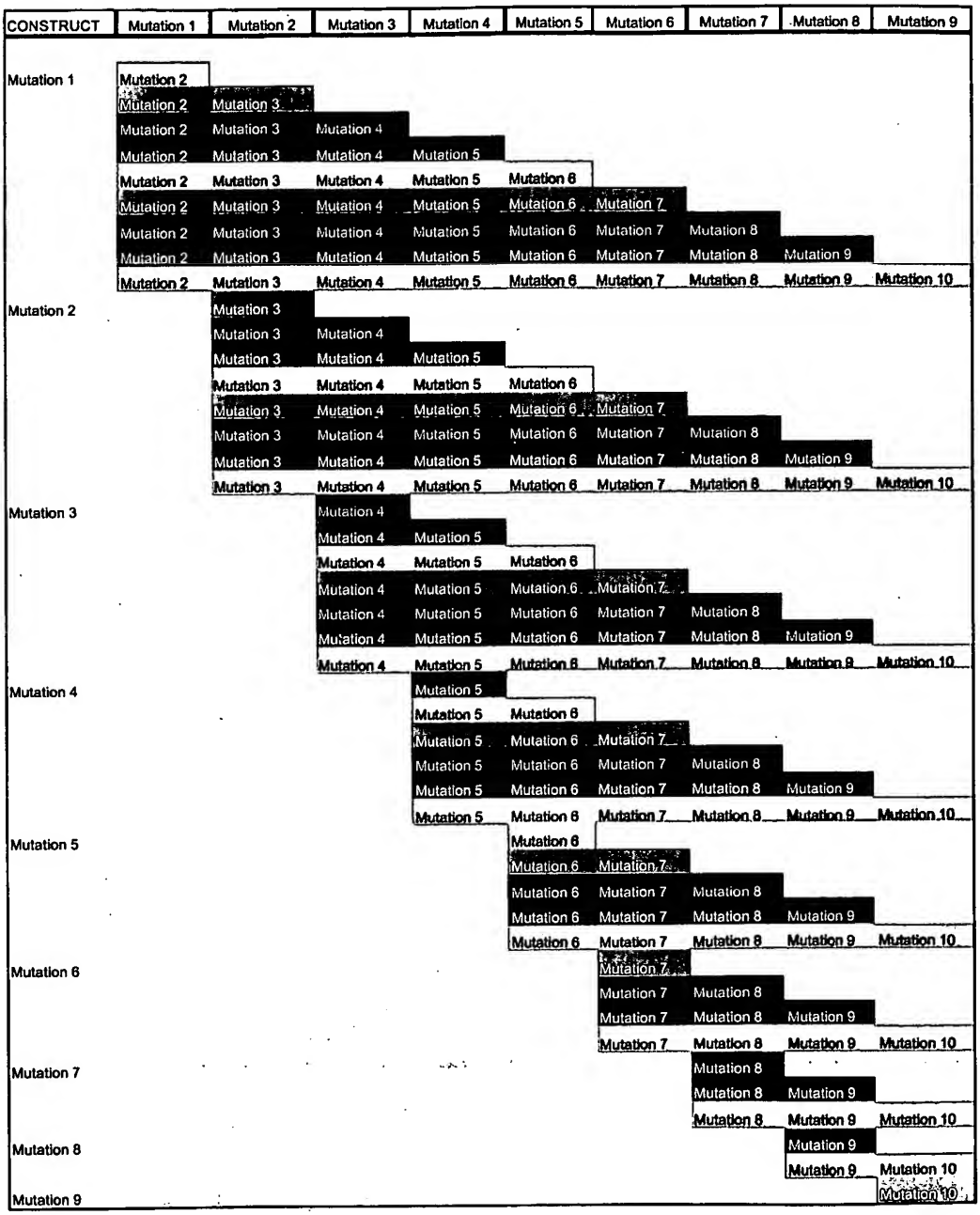

FIG.4

LEADs and SuperLEADs obtained for the Rep protein

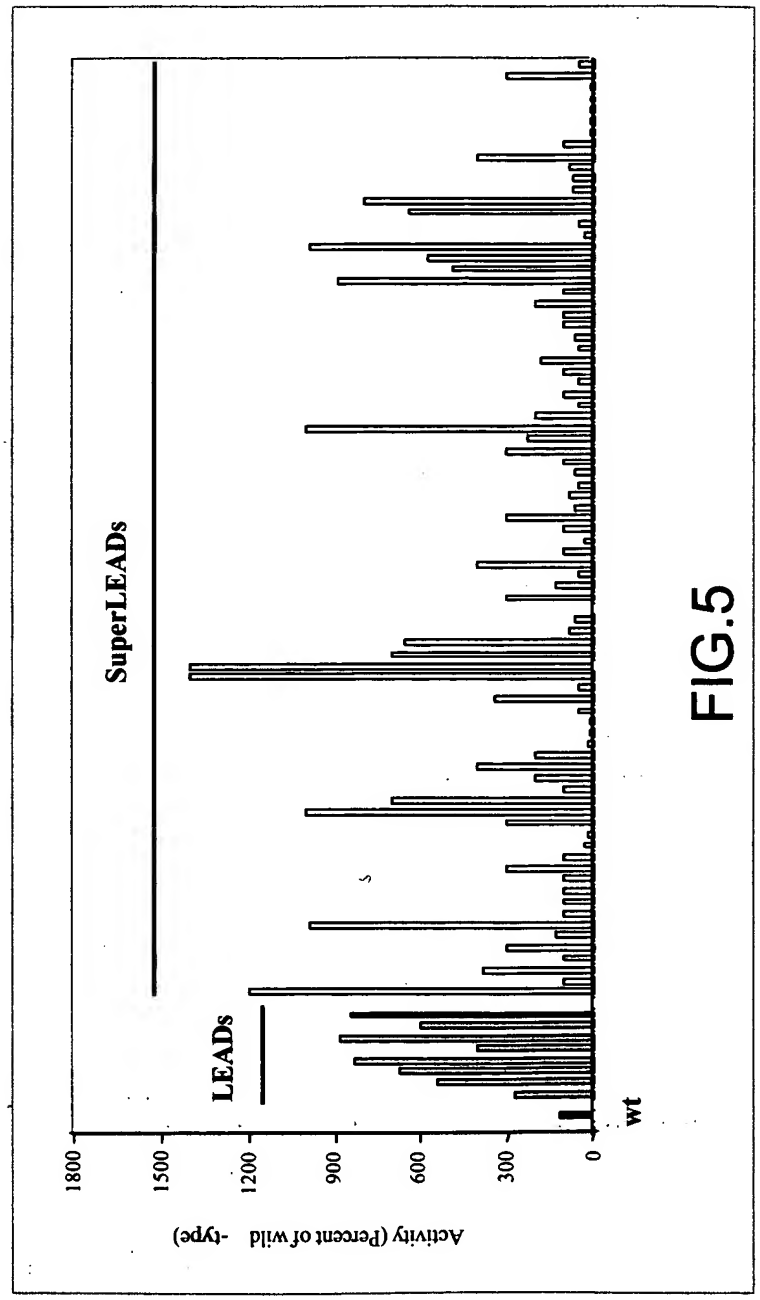

FIG.5

Amino acid sequence of human mature IFNα-2b

```
                1         10         20         30         40         50
                .          .          .          .          .          .
IFNα-2b     CDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKA

               51         60         70         80         90        100
                .          .          .          .          .          .
IFNα-2b     ETIPVLHEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVI

              101        110        120        130        140        150
                .          .          .          .          .          .
IFNα-2b     QGVGVTETPLMKEDSILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRS

              151        160
                .          .
IFNα-2b     FSLSTNLQESLRSKE
```

FIG.6A

REPLACEMENT SHEET
Appln No.: 10/658,355
Applicant(s): Rene Gantier, et. al
RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Three dimensional structure of INFα-2b

showing candidate LEADs

FIG.6B

The "Percent Accepted Mutation" (PAM250) matrix

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	2	-2	0	0	-2	0	0	1	-1	-1	-2	-1	-1	-3	1	1	1	-6	-3	0
R	-2	6	0	-1	-4	1	-1	-3	2	-2	-3	3	0	-4	0	0	-1	2	-4	-2
N	0	0	2	2	-4	1	1	0	2	-2	-3	1	-2	-3	0	1	0	-4	-2	-2
D	0	-1	2	4	-5	2	3	1	1	-2	-4	0	-3	-6	-1	0	0	-7	-4	-2
C	-2	-4	-4	-5	12	-5	-5	-3	-3	-2	-6	-5	-5	-4	-3	0	-2	-8	0	-2
Q	0	1	1	2	-5	4	2	-1	3	-2	-2	1	-1	-5	0	-1	-1	-5	-4	-2
E	0	-1	1	3	-5	2	4	0	1	-2	-3	0	-2	-5	-1	0	0	-7	-4	-2
G	1	-3	0	1	-3	-1	0	5	-2	-3	-4	-2	-3	-5	0	1	0	-7	-5	-1
H	-1	2	2	1	-3	3	1	-2	6	-2	-2	0	-2	-2	0	-1	-1	-3	0	-2
I	-1	-2	-2	-2	-2	-2	-2	-3	-2	5	2	-2	2	1	-2	-1	0	-5	-1	4
L	-2	-3	-3	-4	-6	-2	-3	-4	-2	2	6	-3	4	2	-3	-3	-2	-2	-1	2
K	-1	3	1	0	-5	1	0	-2	0	-2	-3	5	0	-5	-1	0	0	-3	-4	-2
M	-1	0	-2	-3	-5	-1	-2	-3	-2	2	4	0	6	0	-2	-2	-1	-4	-2	2
F	-3	-4	-3	-6	-4	-5	-5	-5	-2	1	2	-5	0	9	-5	-3	-3	0	7	-1
P	1	0	0	-1	-3	0	-1	0	0	-2	-3	-1	-2	-5	6	1	0	-6	-5	-1
S	1	0	1	0	0	-1	0	1	-1	-1	-3	0	-2	-3	1	2	1	-2	-3	-1
T	1	-1	0	0	-2	-1	0	0	-1	0	-2	0	-1	-3	0	1	3	-5	-3	0
W	-6	2	-4	-7	-8	-5	-7	-7	-3	-5	-2	-3	-4	0	-6	-2	-5	17	0	-6
Y	-3	-4	-2	-4	0	-4	-4	-5	0	-1	-1	-4	-2	7	-5	-3	-3	0	10	-2
V	0	-2	-2	-2	-2	-2	-2	-1	-2	4	2	-2	2	-1	-1	-1	0	-6	-2	4

FIG.7

Scores from PAM250, given to residue substitutions to protect human INF α–2b against proteolysis

	R	D	E	L	K	M	F	P	W	Y
A	-2	0	0	-2	-1	-1	-3	1	-6	-3
N	0	2	1	-3	1	-2	-3	0	-4	-2
C	-4	-5	-5	-6	-5	-5	-4	-3	-8	0
Q	1	2	2	-2	1	-1	-5	0	-5	-4
G	-3	1	0	-4	-2	-3	-5	0	-7	-5
H	2	1	1	-2	0	-2	-2	0	-3	0
I	-2	-2	-2	2	-2	2	1	-2	-5	-1
S	0	0	0	-3	0	-2	-3	1	-2	-3
T	-1	0	0	-2	0	-1	-3	0	-5	-3
V	-2	-2	-2	2	-2	2	-1	-1	-6	-2

FIG.8

Residue substitutions expected to allow the creation of a disulfide bond

FIG.9A

FIG.9B

Residue substitutions expected to destroy linking interactions

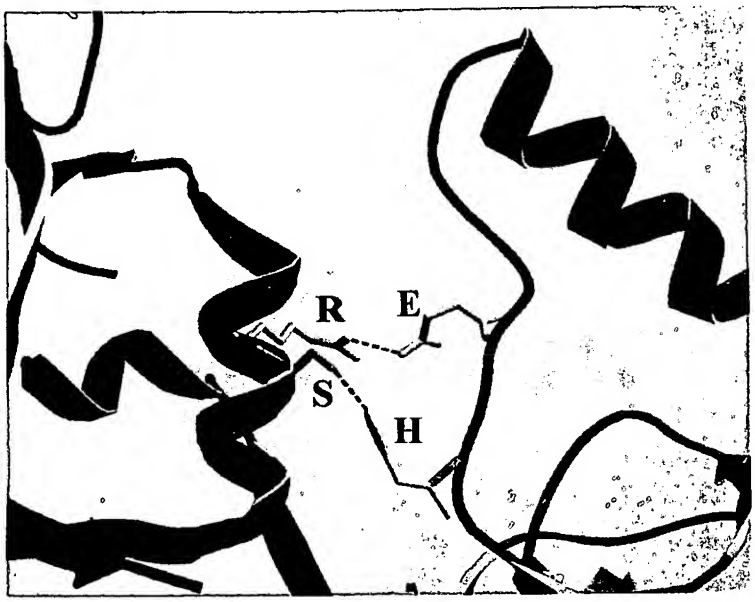

FIG.10A

FIG.10B

Tri-dimensional model of an amphipathic polypeptide

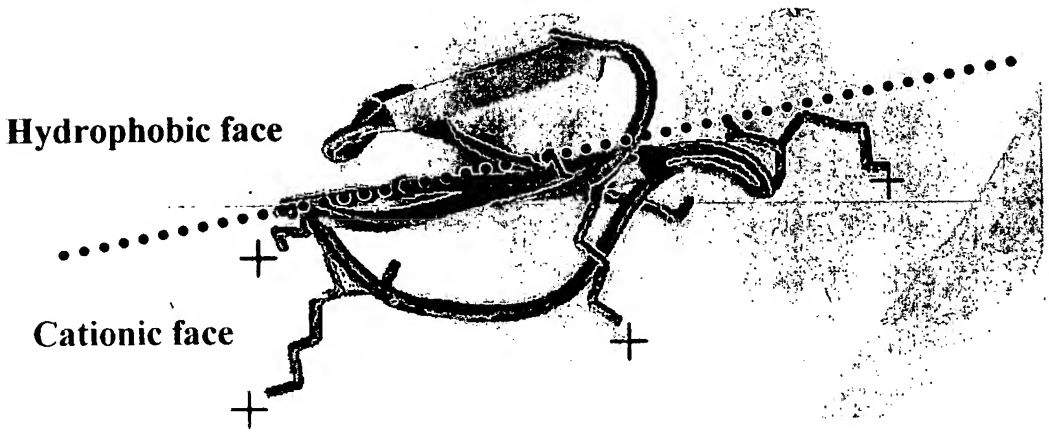

FIG.11

2-D matrix representation of a protein sequence

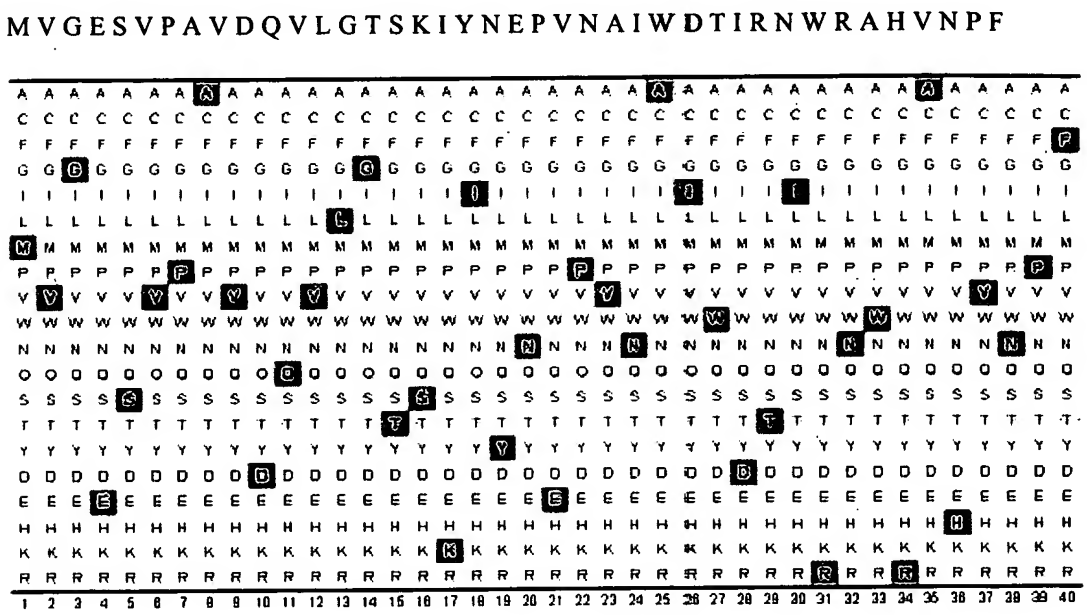

FIG.12

2-D matrix for amphipathic peptide showing K/R scanning: sequential replacement of each residue by either K or R

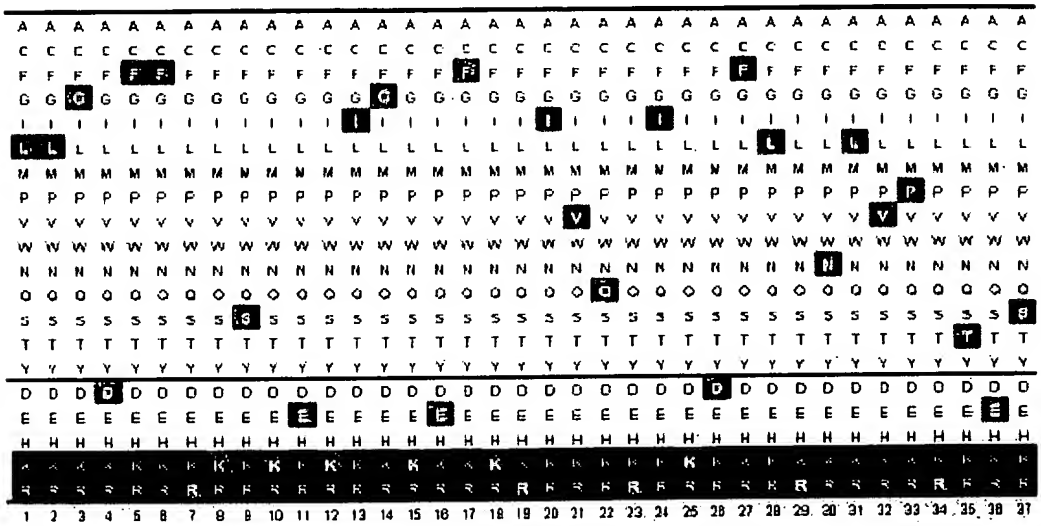

FIG.13A

2-D matrix for K/R scanning on amphipathic polypeptide

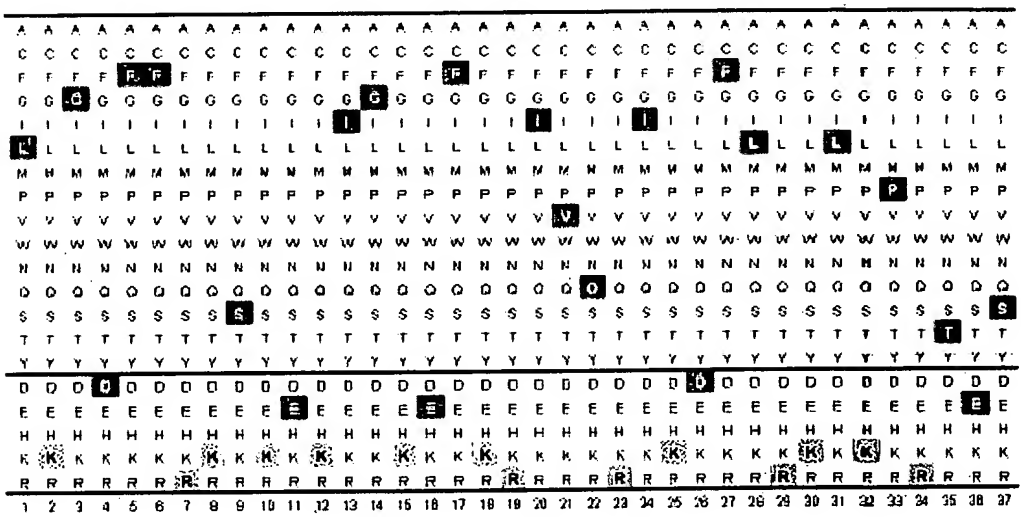

FIG.13B

2-D matrix for LEAD candidates on amphipathic polypeptide

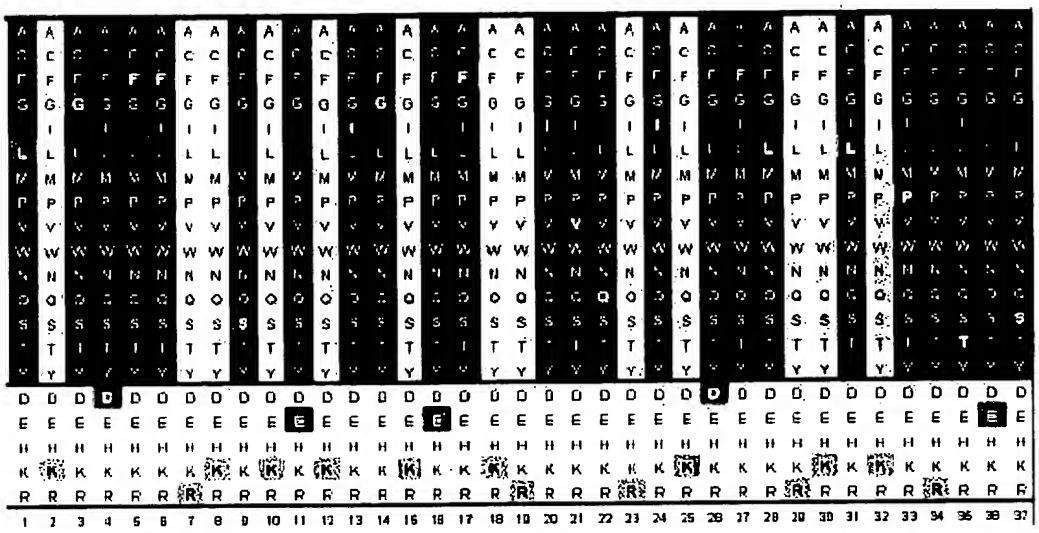

FIG.13C

2-D matrix for optimized amphipathic polypeptide, following both: *i*) K/R scanning (FIG. 13B) and *ii*) mutagenesis (FIG. 13C)

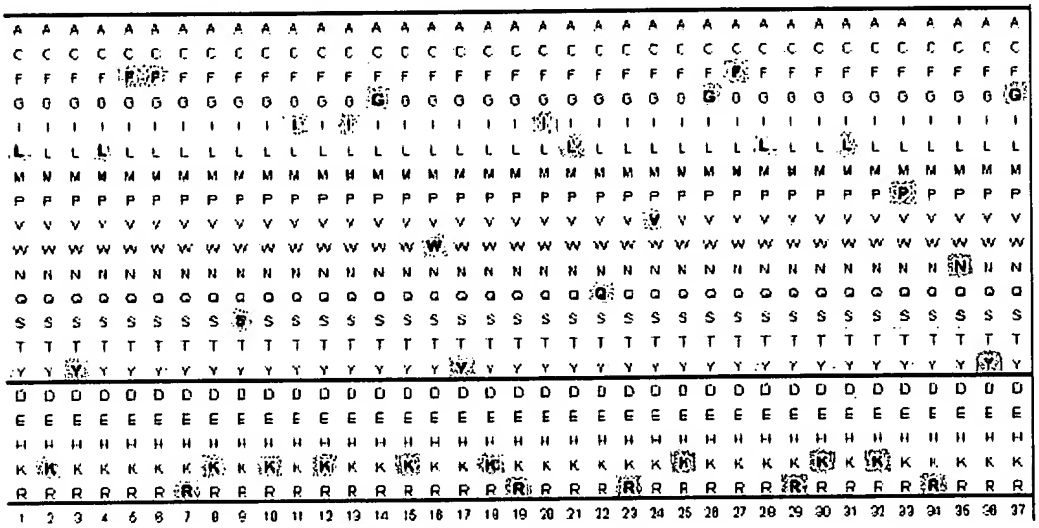

FIG.13D

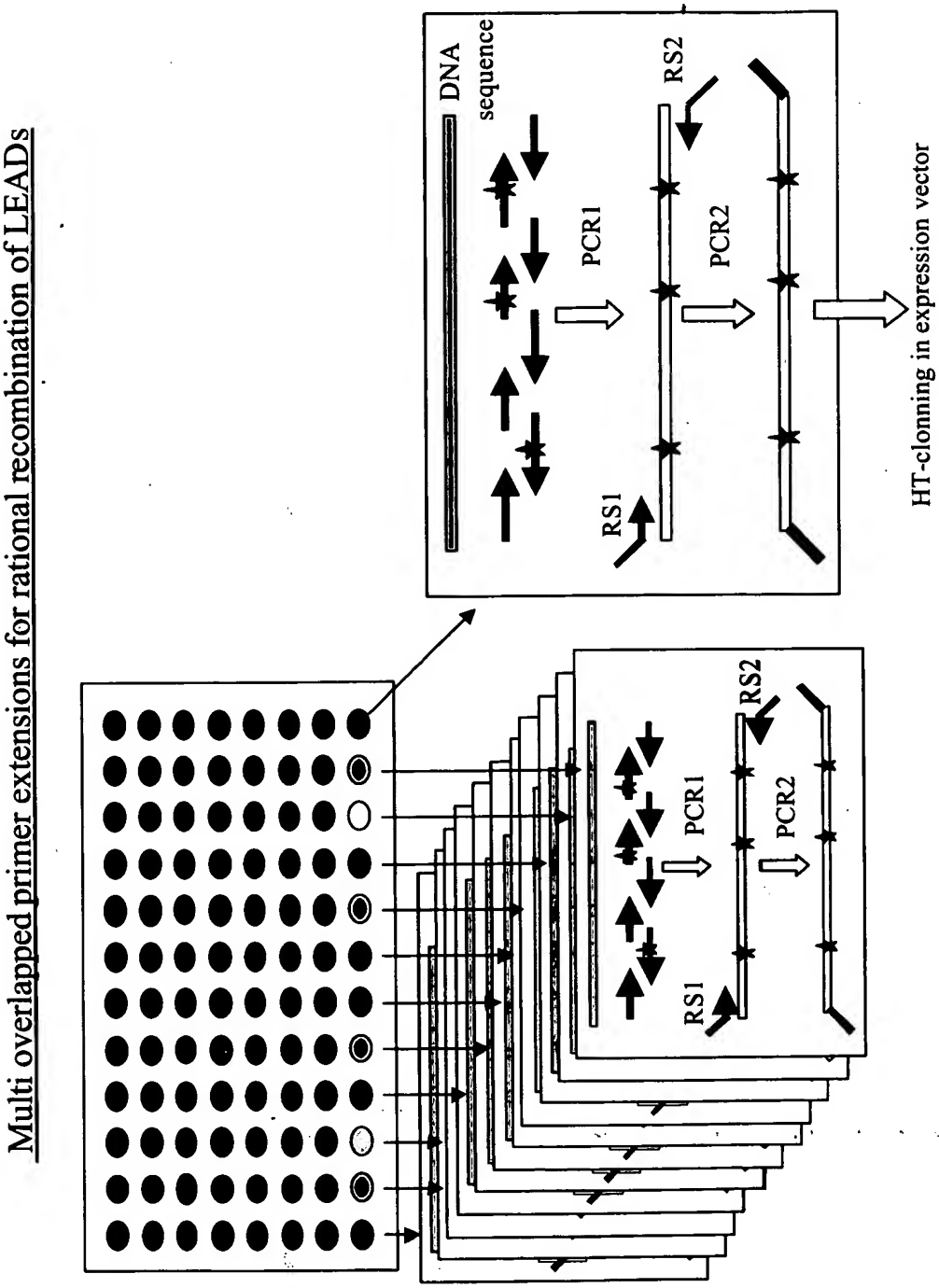
Multi overlapped primer extensions for rational recombination of LEADs
DNA sequence
PCR1
PCR2
RS1
RS2
HT-clonning in expression vector

FIG.14